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RESEARCH ARTICLE

COMPARATIVE STUDY ON HYDROLYTIC ENZYMES PRODUCED BY *Candida albicans* YEAST AND HYPHAL FORM

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ABSTRACT

In recent years, the incidence of fungal infections has been rising all over the world. The ability of Candida albicans to switch from yeast to hyphal growth is essential for its virulence. The aim of this comparative study was to biotype and characterizes phospholipase, proteinase, phosphatase and haemolytic activities of yeast and hyphal form of Candida albicans. The hyphal form of Candida albicans secrets high quantity of hydrolytic enzymes than yeast form which helps in its virulence. These results suggest that pathogenic fungi produce larger amount of inducible hydrolytic enzymes than non pathogenic fungi. In this investigation, we were used plate methods to determine phospholipase, proteinase and haemolytic activities and spectrophotometric method for acid phosphatase activity.

INTRODUCTION

C.albicans is dimorphic organism belongs to the genus *Candida* of ascomycetous like fungal species (Staniszewska *et al* 2012). *C. albican*sis is the major casutive factor of opportunistic human infections with very high morbidity and mortality rate of 30 to 40% (Staniszewska *et al* 2012, Barnett 2008, Bialkova 2006, Bialasiewicz 1996, Biswas*et al* 2000, Borg 1988, Borges-Walmsely 2000, Borman*et al* 2008, Gropper*et al* 2009, Silva *et al* 2010). *Candida albicans* is most virulent *Candida* species that are responsible to cause superficial and systemic infections especially in immunocompromised individuals (Bramono*et al* 2006, Borstt 2003). *Candida albicans* has ability to switch reversibly between a single celled yeast (blastospore) and an elongated filaments form (Hyphae and Pseudohyphae) called as – "Morphological transition or morphological dimorphism" (Tsai *et al* 2013). In *Candida albicans* the yeast to hyphal morphological conversion is well studied and can be induced in vitro with several environmental factors known as "Inducers" (Ghosh *et al* 2009), *Candida albicans* differentially express various infection associated genes, cell surface and virulence proteins which contribute to its pathogenicity and function as "virulence factors" (Tsai *et al*2013). *C.albicans* secretes various hydrolytic enzymes such as acid phosphatases, phospholipases, proteinases, β galactosidase which play important role in candidal outgrowth (Bramono*et al* 2006, Fradin 2003, Bramono et al 1994, Banno*et al* 1985, Ogawa 1997, Naglik*et al* 2004, Tsang *et al* 2007). Hydrolytic enzymes help in adherence, tissue penetration and proliferation of fungi by causing invasion, destruction of host tissues hence supplying degraded material to the organisms as nutrients (Bramono*et al* 2006, Fradin 2003, Ogawa 1997, Naglik*et al* 2004, Tsang *et al* 2007).Seven phospholipase genes have been identified but only four are well characterized (Tsang *et al* 2007, Samaranayake*et al* 2006). Phospholipases contribute to pathogenecity of *C.albicans* by damaging host cell membranes, which helps pathogen to invade host tissues (Borstt 2003). Saps are encoded by 10 SAP genes that play crucial role in *C.albicans* virulence (Tsang *et al* 2007). Proteinases are capable of degrading epithelial and mucosal barrier patients such as collagen, keratin, mucin, antibodies, complement and cytokines (Borstt 2003). Cloning and disruption of the genes for these enzymes showed their role in *Candida* virulence (Borstt 2003, Hube*et al* 1997, Sanglard*et al* 1997, Leidich*et al* 1998, Watts *et al* 1998, De Bernardis*et al* 1999). Acid phosphatase which is located in the cell wall of yeasts (Arnold 1981) belongs to group of periplasmic enzymes secreted by *Candida albicans* (Vasileva- Tonkova*et al* 1993). These enzymes are glycoproteins and their content in yeast cells depend on the phosphate concentration of the growth medium (Vasileva- Tonkova*et al* 1993). Furthermore, Haemolysin is another important virulence factor thought to contribute to candidal pathogenesis (Tsang *et al* 2007). The ability of *C.albicans* to acquire elemental iron through haemolysin production is important in its survival and ability to cause infections in the humans (Tsang *et al* 2007, Weinberg 1978). The secretion of haemolysin, lysis of the erythrocytes, followed by iron acquisition facilitates hyphal invasion in disseminated candidasis (Tsang *et al* 2007, Odds 1998, Rossoni*et al* 2012). Expression of virulence factors helps to understand the epidemiology of infections, which result in improved therapeutic regiments (Borstt 2003). Intensive research is expected to identify pathogenic factors in fungi especially in *Candida albicans* for facilitating the diagnosis, treatment and prevention of candidasis (Bramono*et al* 2006).The objective of this study was to comparatively measured proteinase, lipase, acid phosphatases among yeast and hyphal form of *Candida albicans* and tried to correlate the role of these enzymes in fungal virulence. To our knowledge, the comparative study of different enzyme production by yeast and hyphal form is not yet been published before.

MATERIALS AND METHODS

The study was carried out on two strains MTCC 227 of *Candida albicans* in order to compare the production of different enzymes between yeast form cells (non-pathogenic) and hyphal form cells (pathogenic). This study was performed

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in order to evaluate any possible difference in the secretion of hydrolytic enzymes of different form of *Candida albicans*.

MATERIALS

C. albicans strain

Candida albicans MTCC (ATCC 227), a quality strain was procured from Institute of Microbial Technology (IMTECH), Chandigarh, India and maintained on Yeast extract Peptone Dextrose (YPD) agar slants at 4°C.

Determination of Proteinase activity

Extracellular proteinase activity was measured by using bovine serum activity. BSA as a substrate (Bramono *et al* 2006, Negi et al 1984, Tsuboi *et al* 1985, Tsuboi et al 1989).The activity was analyzed in terms of BSA degradation according to the technique described by Staib *et al* 1965 (Sachin et al 2012, Tsang et al 2007). In this, control (yeast) and test (hyphal) suspension of 1×10^8 cells/ml was prepared, and 200µl suspension was inoculated onto 1%BSA medium

Activity	Yeast Form	Hyphal Form	Activity (Weak-+/Strong-++++)	
	(Value)	(Value)	Yeast Form	Hyphal Form
Phospholipase (Pz)	0.96 ± 0.03	0.69 ± 0.01	+	++++
Proteinase (Prz)	0.94 ± 0.03	0.67 ± 0.01	+	++++
Haemolysin (Hz)	0.86 ± 0.03	0.65 ± 0.01	+	++++

METHODS

Determination of Phospholipase activity

Candida albicans ability to produce extracellular phospholipase activity was determined by measuring zone of precipitation after growth on egg yolk agar (Samaranayake *et al* 1984, Tsang *et al*2007, Sachin *et al*2012). The egg yolk medium consist of 13g Sabouraud's dextrose agar (SDA), 11.7 g NaCl, 0.11g CaCl2 and 10% sterile egg yolk (all in 184 ml distilled water) (Tsang CSP *et al*2007, Mohandas *et al*2011, Sachin *et al*2012). First, the components without the egg yolk were mixed and sterilized, then the egg yolk was centrifuged at 500g for 10min at room temperature and 20ml of the supernatant was added to the sterilized medium (Sachin *et al*2012, Tsang *et al*2007).

Standard inocula of the test (hyphal) and control (yeast) Candida (5µl with 10^8 yeast cells were deposited onto the egg yolk agar medium and kept at room temperature (Sachin *et al*2012, Tsang*et al* 2007). Each culture was then incubated at 37°C for 48h (Sachin *et al*2012, Tsang*et al* 2007, Mahmoudabadi*et al* 2010) after which the diameter of precipitation zone around the colony was determined (Sachin *et al*2012, Tsang *et al*2007, Mahmoudabadi*et al* 2010). Phospholipase activity (Pz value) was determined by taking ratio of the diameter of the colony plus the precipitation zone (in mm) Price *et al* 1982 method (Sachin *et al*2012, Tsang *et al*2007, Mahmoudabadi*et al* 2010, Ying *et al* 2012).

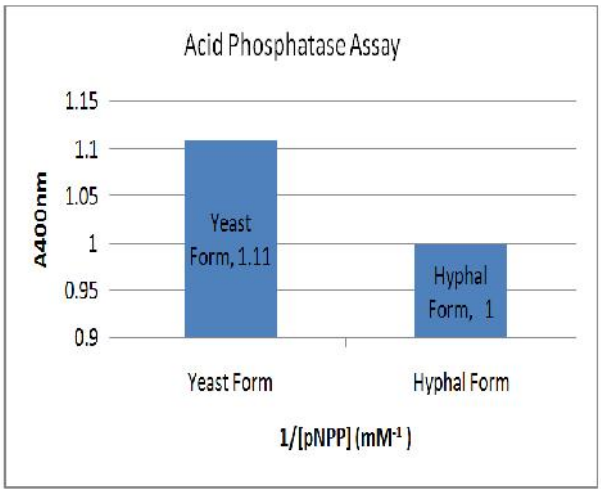
$$\frac{\textit{Colony diameter}}{\textit{Colony diameter + Zone of precipitation}} = Pz$$

Phospholipase activity of the isolate was considered positive when a precipitation zone was observed around the colony on the plate (Sachin *et al*2012, Tsang *et al*2007, Mahmoudabadi*et al* 2010, Ying*et al 2012*). Pz value equals to 1 denotes no activity or negative for phospholipases, less than 1 (Pz<1) indicates phospholipase activity. The lower the Pz value, higher is the enzymatic activity (Ying *et al* 2012). Pz<0.90-0.99=weak phospholipase activity (+), 0.80-0.89= poor phospholipase activity (++), 0.70-0.79= moderate phospholipase activity (+++) and Pz < 0.70 = large phospholipase activity (++++) (Sachin *et al* 2012, Tsang *et al* 2007, Mahmoudabadi*et al* 2010). Reference strains of *Candida albicans* (ATCC10231 and ATCC 24433) were taken as positive control (Sachin *et al* 2012, Tsang *et al* 2007).

Statistical analysis: The assay was carried out in triplicate.

plate (2% glucose, 0.1% KH2PO4,0.05% MgSo4 2% agar mixed after cooling to 50 °C with 1%BSA solution)(Sachin *et al* 2012, Tsang *et al* 2007). The plate was incubated for 5 days at 37 °C, after which the diameter of precipitation zone around the well was determined which indicates proteinase activity (Sachin *et al* 2012, Tsang *et al* 2007). Proteinase activity (Prz) was determined as the ratio of the diameter of the colony to that of the clear zone of proteolysis (in mm) (Sachin *et al* 2012, Tsang *et al* 2007, Akcaglar et al 2010). Reference strain of *Candida albicans* (ATCC 10231 and ATCC 10261) were taken as positive control (Sachin *et al* 2012, Tsang *et al* 2007).

Statistical analysis: The assay was carried out in triplicate.



Determination of Haemolys in activity

Hemolysin production was evaluated according to Manns *et al* method (Rossoni *et al* 2013, Sachin *et al* 2012, Tsang *et al* 2007, Manns *et al* 1994, Luo *et al* 2001). Media was prepared by adding 7ml fresh sheep blood to 100ml SDA supplemented with glucose at a final concentration of 3% (w/v). The final pH of the medium was 5.6±0.2 (Sachin *et al* 2012, Tsang *et al* 2007). The culture of both control (yeast) and test (hyphal) of *Candida albicans* (200µl, 108 cells/ml saline) was inoculated into the well in the medium. The plate was then incubated at 37°C in 5% CO_2 for 48h (Sachin *et al* 2012, Tsang *et al* 2007, Rossoni*et al* 2013). After incubation, plates were examined and quantification of colonies was done. The haemolytic index (Hz value) was used to determine haemolysin activity of hyphal and non hyphal cells (Sachin *et al* 2012, Tsang *et al* 2007). Hz was calculated by the ratio of the diameter of the colony to that of the translucent zone of haemolysis in mm

(Sachin *et al* 2012, Tsang *et al* 2007, Rossoni*et al* 2013). A reference strain of *Candida albicans* (ATCC 90028) was taken as a positive control (Sachin *et al* 2012, Tsang *et al* 2007).

Statistical analysis: The assay was carried out in triplicate.

Determination of Acid phosphatase activity

Determination of Acid phosphatase activity by *Candida albicans* was carried out with *p*-nitrophenyl phosphate (pNPP) as a substrate (Vasileva-Tonkova*et al* 1992). The reaction mixture contained 100µl enzyme sample, 100µl 0.1M-sodium acetate buffer (pH 5.5) and 100µl 3.8mM- pNPP (Vasileva-Tonkova*et al* 1992). After incubation at 37 ^{0}C for 15 min, the reaction was stopped by addition of 1ml 0.2M NaOH (Vasileva- Tonkova*et al* 1992). The absorbance was measured at A400 (Vasileva- Tonkova*et al* 1992). One unit of phosphotase activity was defined as the amount of enzyme catalysing the formation of 1µmol p-nitrophenol/min under standard assay conditions (Vasileva-Tonkova*et al* 1992). When some other substrate was used, the assay is carried out according to Lanzeta*et al* 1979 method (Vasileva-Tonkova*et al* 1992).

RESULT &DISCUSSION

Phospholipase, Proteinase and Haemolysin Assay

Acid Phosphatase Assay

For, acid phosphatase activity when reading was taken at 400nm it was found to be 1.11 for acid phosphatase and 1 for hyphal form.

Result and discussion

The pathogenicity of Candida albicans depends on several virulence factors, including germination, adherence to host cells, phenotypic switching and production of extracellular enzymes (Sachin et al 2012). In this comparative study, it was found that hyphal form produces higher amount of phospholipase, proteinase, haemolysin and acid phosphatase enzyme as compared to yeast form. It supports the data that these enzymes also plays role in the pathogenicity of C.albicans and helps for their virulence. To the best of our knowledge, this is first attempt to compare the enzyme secretion between yeast and hyphal form of Candida albicans.

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